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Effect of clofibrate administration on the esterification and deesterification of steroid hormones by liver and extrahepatic tissues in rats

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Abstract

Treatment of rats with clofibrate markedly stimulated the liver microsomal esterification of estradiol, testosterone, pregnenolone, dehydroepiandrosterone, and corticosterone by acyl-CoA:steroid acyltransferase. This enzyme catalyzes the esterification of estradiol with long-chain fatty acids in both liver and extrahepatic tissues. In untreated control rats, brain had the highest acyltransferase activity per milligram of microsomal protein for estradiol esterification (3- to 4-fold higher than in the liver). Although, treatment of rats with clofibrate stimulated the esterification of estradiol by 9- to 14-fold in the liver, estradiol esterification in kidney, lung, brain, uterus, fat, and mammary glands was not increased, indicating that liver may be uniquely sensitive to induction of acyl-CoA:estradiol acyltransferase by clofibrate. In additional studies, esterase activity for hydrolysis of the oleoyl ester of estradiol was determined in control and clofibrate-treated rats. Clofibrate administration increased esterase activity by an average of 107% in fat and 70% in liver. The results indicate that treatment of rats with clofibrate stimulates the hepatic formation of highly lipophilic fatty acid esters that can be hydrolyzed in the liver and in extrahepatic tissues to the parent steroid hormone by a clofibrate-inducible esterase.

Keywords: Synthesis and metabolism of steroid esters; Fatty acid esters of steroid hormones; Deesterification of steroid fatty acid esters; Acyl-CoA:estradiol acyltransferase; Induction of estradiol esterification; Induction of testosterone esterification

1. Introduction

In an earlier study, we found that treatment of rats with clofibrate stimulated by many-fold the liver microsomal esterification of estradiol with several long-chain fatty acids [1]. Since the fatty acid esters of estradiol are very lipophilic and have long half-lives [2–4], they would be expected to concentrate in fat, where release of free estradiol by the action of an esterase [3] could enhance the availability of estradiol in target tissues. Accordingly, we hypothesized that administration of clofibrate to rats would increase the action of estradiol in the mammary gland, a fat-containing tissue. Indeed, administration of clofibrate to rats enhanced the action of estradiol in the mammary glands but not in the uterus [5]. In the present study, we evaluated the effect of treatment of rats with clofibrate on the *in vitro* esterification and deesterification of steroid hormones by liver and extrahepatic tissues. The results indicate that treatment of rats with clofibrate stimulates the formation of highly lipophilic steroid-fatty acid esters that can be hydrolyzed to the parent steroid hormone by a clofibrate-inducible esterase.

2. Materials and methods

2.1. Chemicals

[2,4,6,7,16,17-^{3}H(N)]Estradiol (110–170 Ci/mmol), [4-^{14}C]estrone, [1,2,6,7-^{3}H(N)]testosterone (85–105 Ci/mmol), [7-^{3}H(N)]pregnenolone (10–25 Ci/mmol), [1,2,6,7-^{3}H(N)]dehydroepiandrosterone (70–100 Ci/mmol), and [1,2,6,7-^{3}H(N)]corticosterone (70–100 Ci/mmol) were purchased from Du-Pont New England Nuclear Life Science Products, Inc. Estradiol, estrone, testosterone,

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pregnenolone, dehydroepiandrosterone, corticosterone, clofibrate, and oleoyl-CoA were purchased from Sigma. All solvents were of HPLC grade and were purchased from Fisher Scientific.

2.2. Animals

Female Sprague–Dawley rats (8 weeks old) were obtained from the Harlan Sprague–Dawley Laboratory. The animals were fed AIN-76A diet or 0.5% clofibrate in AIN-76A diet for 2 weeks. All diets were obtained from Research Diets Inc. Liver, mammary glands (abdominal pair), and abdominal fat pads were removed. In a second experiment, the rats were fed 0.6% clofibrate in AIN-76A diet for 4 weeks. Liver, kidney, uterus, lung, and brain (cortex, cerebellum, and brain stem) were collected. Liver microsomes and cytosol were prepared as described previously [6]. Some procedural modifications were adopted for the preparation of microsomal and cytosolic fractions from abdominal fat pads and mammary glands. These tissues were first ground in liquid nitrogen to obtain small particles and then were homogenized in 2.5 vol. of the homogenizing buffer (0.05 M Tris–HCl, 1.15% KCl, pH 7.4) with a polytron homogenizer. The homogenate was centrifuged at 9000 *g* for 30 min at 4°, and a thick layer of fat on top of the supernatant was discarded. The supernatant was centrifuged further at 105,000 *g* for 90 min at 4°. The supernatant (cytosol) from the second centrifugation was filtered to remove additional fat, and the pellet (microsomes) was resuspended in 0.25 M sucrose.

2.3. Assay for the esterification of estradiol, testosterone, dehydroepiandrosterone, pregnenolone, and corticosterone with oleic acid

The structures of estradiol, testosterone, dehydroepiandrosterone, pregnenolone, and corticosterone, as well as the sites of fatty acid esterification, are shown in Fig. 1. Enzyme assays for the esterification of estradiol and the other steroids were carried out essentially as described in one of our recent studies [1]. Incubation mixtures contained 50 μM ^{3}H-labeled estradiol, testosterone, dehydroepiandrosterone, pregnenolone, or corticosterone (1–5 μCi) or 50 μM ^{14}C-labeled estrone (0.3 μCi) as well as 100 μM oleoyl-CoA and 5 mM magnesium chloride in 0.1 M sodium acetate buffer (pH 5.0). The reaction was initiated by the addition of liver microsomes (0.25–0.5 mg protein), and the final volume of the incubation mixture was 0.5 mL. After incubation at 37° for 30 min, the reaction was arrested by placing the tubes on ice, followed by the addition of 0.5 mL of ice-cold sodium acetate buffer (pH 5.5) and 5 mL of ethyl acetate (HPLC grade). The samples were vortexed immediately and centrifuged for 10 min at 3000 *g* at 4°. The upper ethyl acetate phase was removed, and the aqueous phase was extracted a second time. The organic solvent extracts were combined and evaporated to dryness under a stream of nitrogen. Each resulting residue was dissolved in 100 μL of methanol and analyzed by HPLC.

Measurement of esterified metabolites of estradiol, testosterone, dehydroepiandrosterone, pregnenolone, and corticosterone was done by HPLC with a Spherisorb ODS

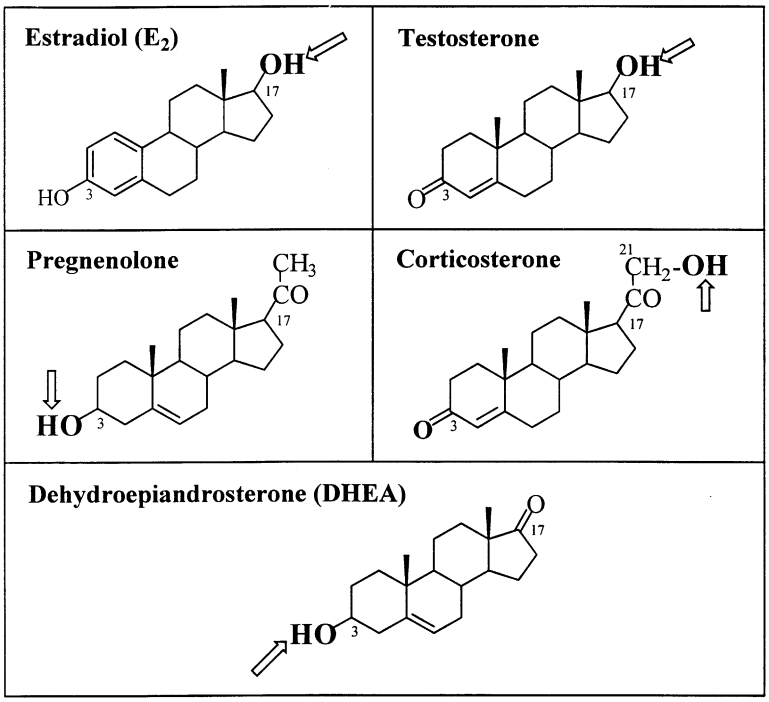

Fig. 1. Structures of several steroids and sites of fatty acid esterification. The site of esterification is indicated by an arrow.

column (5 μm particle size, 250 mm×4.6 mm i.d.). The HPLC system consisted of a Waters 600E solvent gradient programmer, a Waters Lambda-Max model 481 UV detector, and a radioactive flow detector (β-ram from IN/US), as described [1]. The solvent system for the oleoyl ester metabolites of estradiol and corticosterone consisted of: acetonitrile−/−0.1% acetic acid in H_2O−/−methanol. The solvent gradient used for elution of the oleoyl ester of estradiol from the column was: 12 min isocratic at 30/6/64; 6 min with a number 10 convex gradient to 60/0/40; 15 min isocratic at 60/0/40; 2 min with a number 2 convex gradient to 20/0/80; 5 min isocratic at 20/0/80, and the column was then returned to initial conditions over 15 min. The same gradient was used in studies with estrone. The number 2 and 10 convex gradients are described in the brochure associated with the Waters 600E solvent gradient programmer. The solvent for the elution of the oleoyl ester of corticosterone was isocratic at 30/6/64. The solvent for elution of the oleoyl ester of testosterone, pregnenolone, or dehydroepiandrosterone from the column was isocratic at 100% methanol. The flow rate was 1.2 mL/min. The HPLC chromatographic separation of the oleoyl esters of estradiol, testosterone, pregnenolone, dehydroepiandrosterone, or corticosterone from their respective parent steroid is shown in Fig. 2. Metabolite quantification was based on the amount of radioactivity associated with the metabolite peak as compared to the total radioactivity eluted from the HPLC column from each sample.

2.4. Esterase activity assay

^{3}H-Labeled estradiol-oleoyl ester was used as the substrate for esterase assays. Since radiolabeled estradiol fatty acid esters were not available commercially, ^{3}H-labeled estradiol-oleoyl ester was enzymatically synthesized. [^{3}H]Estradiol (50 μM) was incubated with liver microsomes from rats treated with clofibrate (to increase the esterification rate) and oleoyl-CoA in sodium acetate buffer (pH 5.0) as described above except that the incubation volume was increased. After extractions with ethyl acetate, the samples were dried under nitrogen, dissolved in methanol, and injected into the HPLC. Estradiol-oleoyl ester was isolated using the HPLC conditions described above. To check the purity of the isolated estradiol-oleoyl ester, a small portion was reinjected into the HPLC system. Only one peak was observed, and the retention time of the peak corresponded to estradiol-oleoyl ester. The total radioactivity of ^{3}H-labeled estradiol-oleoyl ester collected

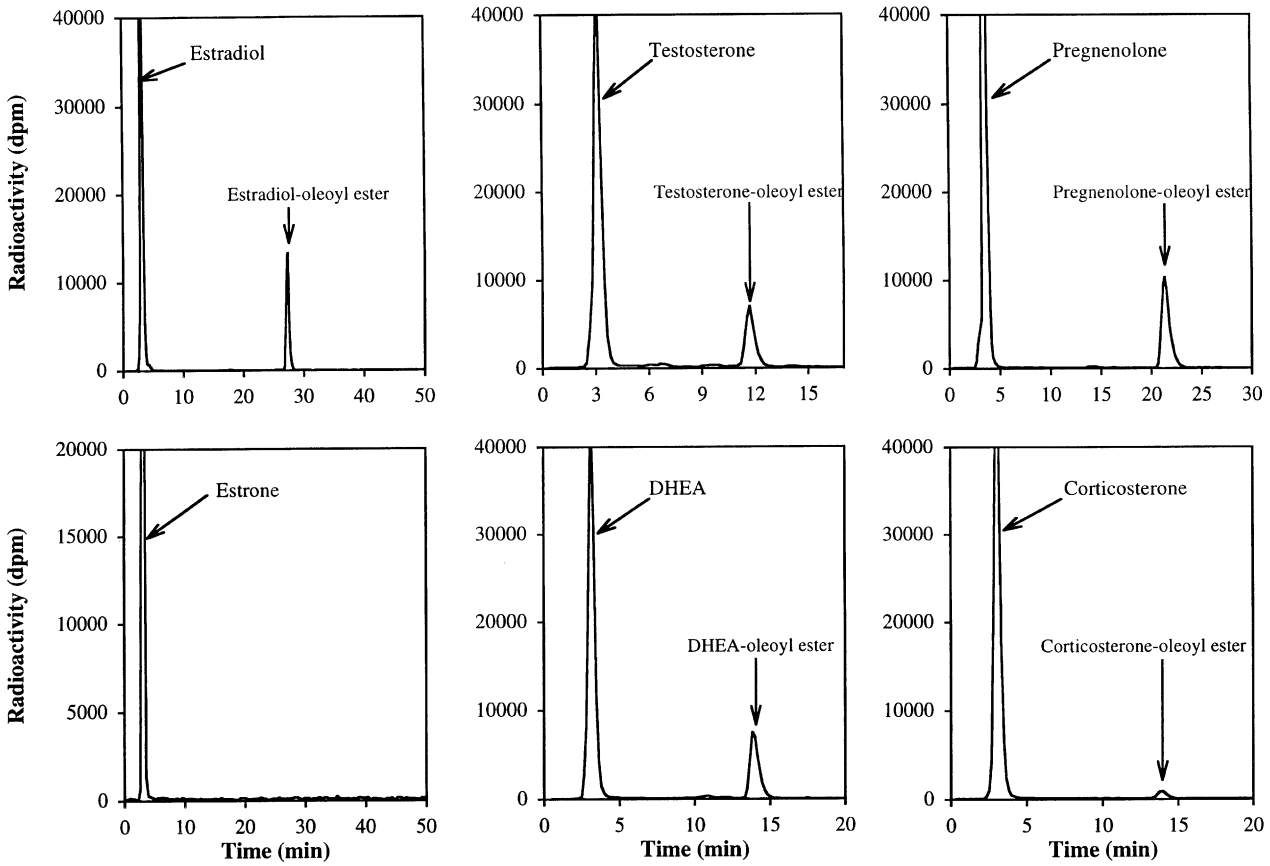

Fig. 2. Metabolism of estradiol, testosterone, pregnenolone, dehydroepiandrosterone (DHEA), and corticosterone to their oleoyl esters (HPLC chromatography). ^{3}H-Labeled steroids (50 μM) were incubated with oleoyl-CoA (100 μM), magnesium chloride (5 mM), and rat liver microsomes (1 mg protein/mL) at pH 5.0 for 30 min, and steroid oleoyl ester was measured by HPLC as described in Section 2. This particular experiment was performed only once, but similar results were obtained for the multiple incubations described in Table 1.

and its specific radioactivity were calculated. The concentration of estradiol-oleoyl ester used as a substrate for esterase activity measurements was determined from the radioactivity of the estradiol-oleoyl ester formed.

The reaction mixture for esterase assays consisted of 3.3 μM ^{3}H-labeled estradiol-oleoyl ester (~0.4 μCi) and 1 mg protein/mL of the subcellular fractions (microsomes or cytosol) in a total volume of 200 μL of phosphate buffer (pH 7.5). After incubation at 37° for 30 min, the reaction was arrested by placing the tubes on ice, followed by the addition of ice-cold phosphate buffer (pH 7.5) and 2 mL of ethyl acetate. The samples were vortexed immediately and centrifuged for 10 min at 3000 *g* at 4°. The organic phase was removed, and the extraction was repeated a second time. The organic solvent extracts were combined and evaporated to dryness under a stream of nitrogen. Each resulting residue was dissolved in 100 μL of methanol and analyzed by HPLC.

The solvent gradient used for the elution of estradiol and the oleoyl ester of estradiol from the column using acetonitrile−/−0.1% acetic acid in H_2O−/−methanol was as follows: 12 min isocratic at 30/6/64; 6 min with a number 10 convex gradient to 60/0/40; 15 min isocratic at 60/0/40; 2 min with a number 2 convex gradient to 20/0/80; 5 min isocratic at 20/0/80, and the column was then returned to initial conditions over 15 min. The retention times for estradiol and estradiol-oleoyl ester were 3 and 27.6 min, respectively. For esterase activity assays, an estradiol peak was detected after incubation of estradiol-oleoyl ester with the subcellular fractions, and quantification was based upon the amount of radioactivity in the estradiol peak as compared with the total radioactivity eluted from the HPLC column.

2.5. Statistical analysis

Statistical analyses were done by Student's *t*-test.

3. Results

3.1. Esterification of estradiol, testosterone, dehydroepiandrosterone, pregnenolone, and corticosterone by liver microsomes from female Sprague–Dawley rats

In this study, we determined the *in vitro* esterification of several steroids by liver microsomes from control untreated Sprague–Dawley rats. Estradiol, testosterone, dehydroepiandrosterone, pregnenolone, and corticosterone (50 μM) were esterified to oleoyl esters when incubated with liver microsomes and oleoyl-CoA (Fig. 2). The acyltransferase reaction was approximately linear with time of incubation up to 30 min and was proportional to microsomal protein concentration from 0.25 to 1 mg protein/mL of incubation mixture for liver microsomes from control or clofibrate-treated animals (data not presented). The acyltransferase activity in liver microsomes for estradiol esterification was the highest (53.2 pmol/mg protein/min), followed by esterification of testosterone (34.0 pmol/mg protein/min), dehydroepiandrosterone (27.9 pmol/mg protein/min), pregnenolone (23.6 pmol/mg protein/min), and corticosterone (2.8 pmol/mg protein/min) (Table 1). Esterification of corticosterone was only 1/20th of that for estradiol (Table 1). Estrone was not esterified by rat liver microsomes (Table 1, Fig. 2).

3.2. Effect of clofibrate administration on the liver microsomal esterification of estradiol, testosterone, dehydroepiandrosterone, pregnenolone, and corticosterone

Female Sprague–Dawley rats were fed 0.6% clofibrate diet for 4 weeks. The esterification of estradiol, testosterone, dehydroepiandrosterone, pregnenolone, and corticosterone by liver microsomes was stimulated by 14-, 17-, 22-, 21- and 22-fold, respectively (Table 1). As was observed for liver microsomes from control rats, liver microsomes from clofibrate-treated rats esterified estradiol to a greater extent than the other steroids (738.3 pmol/mg protein/min), and corticosterone was esterified the least (61.7 pmol/mg protein/min) (Table 1). Liver microsomes from clofibrate-treated rats did not esterify estrone (Table 1).

3.3. Effect of clofibrate administration on the esterification of estradiol in extrahepatic tissues

Female Sprague–Dawley rats were fed 0.5% clofibrate diet for 2 weeks. The microsomal esterification of estradiol

Table 1
Stimulatory effect of treatment of rats with clofibrate on the liver microsomal metabolism of estradiol, testosterone, dehydroepiandrosterone, pregnenolone, and corticosterone to their oleoyl esters

Steroid incubated	Formation of steroid-oleoyl ester (pmol/mg protein/min)	
	Control diet	Clofibrate diet
Estradiol	53.2 ± 2.6	738.3 ± 45.1
Testosterone	34.0 ± 1.8	580.3 ± 21.9
Dehydroepiandrosterone	27.9 ± 0.5	609.9 ± 18.3
Pregnenolone	23.6 ± 1.0	491.6 ± 25.8
Corticosterone	2.8 ± 0.2	61.7 ± 4.7
Estrone	ND [a]	ND

Female Sprague–Dawley rats were fed AIN-76A diet or 0.6% clofibrate in AIN-76A diet for 4 weeks. The animals were killed and livers were removed for the preparation of microsomes. The incubation mixture consisted of 0.5 mg/0.5 mL of microsomal protein from control rats or 0.25 mg/0.5 mL of microsomal protein from treated rats, 50 μM ^{3}H-labeled steroid (estradiol, testosterone, dehydroepiandrosterone, pregnenolone, or corticosterone) (1–2 μCi) or ^{14}C-labeled estrone (~0.28 μCi), and 100 μM oleoyl-CoA in a final volume of 0.5 mL of sodium acetate buffer (0.1 M, pH 5.0). Each value is the mean ± SEM obtained from microsomes from six rats.

[a] ND: not detected.

Table 2
Effect of clofibrate administration on microsomal acyl-CoA:estradiol acyltransferase activity in liver and extrahepatic tissues

Tissue	Formation of estradiol-oleoyl ester (pmol/mg protein/min)	
	Control diet	Clofibrate diet
Experiment 1		
Liver	42.5 ± 2.2	383.7 ± 31.0*
Fat	60.2 (62.5, 58.0)	57.2 (56.2, 58.3)
Mammary glands	18.2 (21.4, 14.9)	20.6 (23.8, 17.5)
Experiment 2		
Liver	53.2 ± 2.6	738.3 ± 45.1*
Kidney	26.3 ± 1.3	32.1 ± 2.4
Lung	84.5 ± 3.8	103.6 ± 5.6
Brain cortex	173.4 ± 10.1	182.7 ± 24.9
Brain cerebellum	224.9 ± 29.5	272.8 ± 45.8
Brain stem	220.7 ± 10.1	205.6 ± 8.0
Uterus	57.6 ± 2.8	52.8 ± 6.1

In experiment 1, female Sprague–Dawley rats were fed AIN-76A diet or 0.5% clofibrate in AIN-76A diet for 2 weeks. Liver, the abdominal pair of mammary glands, and the abdominal fat pads were removed for the preparation of microsomes. The incubation mixture consisted of microsomal protein (0.5 or 0.25 mg/0.5 mL), 50 μM estradiol, and 100 μM oleoyl-CoA in sodium acetate buffer (pH 5.0). Incubations were for 30 min. The formation of estradiol-oleoyl ester was measured as described in Section 2. Each value is the mean ± SEM obtained from four to five rats for liver samples or the mean of two values (each obtained from pooled tissues from five rats) for the extrahepatic tissues. The individual values are indicated in parentheses. In experiment 2, rats were fed AIN-76A diet or 0.6% clofibrate in AIN-76A diet for 4 weeks. The formation of estradiol-oleoyl ester was measured as in experiment 1. Each value is the mean ± SEM from five to six rats (for liver, kidney, and lung) or from three pooled samples (each from eight to nine rats) for brain and the uterus.

* $P < 0.001$ in comparison with the control diet fed group.

was determined in liver, mammary glands (abdominal pair), and abdominal fat pads (Table 2). Fatty acyl-CoA:estradiol acyltransferase activity in fat tissue was higher than that in the mammary glands. Since the mammary glands contain adipocytes in addition to mammary glandular cells, the lower level of acyltransferase activity in mammary glands in comparison with fat suggested the possibility of low acyltransferase activity in glandular cells. Administration of clofibrate had a strong stimulatory effect on the microsomal esterification of estradiol in liver, but this treatment had no significant effect on estradiol esterification in fat tissue or in the mammary glands (Table 2, experiment 1).

In an additional study, female Sprague–Dawley rats were fed 0.6% clofibrate diet for 4 weeks. Microsomal esterification of estradiol was measured in liver and in extrahepatic tissues, including kidney, lung, uterus, brain cortex, brain cerebellum, and brain stem (Table 2, experiment 2). Among the extrahepatic tissues studied, kidney had the lowest acyl-CoA:estradiol acyltransferase activity (26.3 pmol/mg/min), and brain had the highest acyl-CoA:estradiol acyltransferase activity (173.4–224.9 pmol/mg/min). The three parts of the brain—cortex, cerebellum, and brain stem—had similar acyltransferase activity for estradiol esterification. Although estradiol esterification by liver microsomes was stimulated 14-fold in rats treated with clofibrate, acyl-CoA:estradiol acyltransferase activity in extrahepatic tissues was not stimulated by clofibrate administration (Table 2).

3.4. Effect of clofibrate administration on esterase activity in liver and extrahepatic tissues

Esterase activity for the hydrolysis of estradiol fatty acid esters was determined by measuring the formation of free estradiol from the oleoyl ester of estradiol. Since radioactive estradiol fatty acid esters were not available commercially, we prepared ^{3}H-labeled estradiol-oleate biosynthetically (Section 2), and we isolated the product by HPLC. The hydrolysis of the oleoyl ester of estradiol was approximately linear with incubation time up to 30 min, and the esterase activity was higher at pH 7.5 than at pH 5.0 (optimum pH for acyl-CoA:estradiol acyltransferase) (data not presented). In esterase assays, we used a 30 min incubation in phosphate buffer at pH 7.5. Both microsomal and cytosolic fractions had comparable

Table 3
Effect of clofibrate administration on estradiol fatty acid ester esterase activity in liver and extrahepatic tissues

Tissue	Subcellular fraction	Formation of estradiol (pmol/mg protein/hr)		Percent increase
		Control diet	Clofibrate diet	
Liver	Cytosol	13.6 ± 1.4	25.2 ± 4.8*	85
	Microsomes	11.0 ± 0.4	16.9 ± 0.6**	54
Fat	Cytosol	33.6 (40.2, 27.1)	63.8 (68.2, 59.4)	90
	Microsomes	33.9 (40.1, 27.6)	76.2 (77.7, 74.7)	124
Mammary glands	Cytosol	34.7 (38.0, 31.4)	42.1 (39.5, 44.7)	21
	Microsomes	27.9 (27.8, 28.0)	45.5 (44.2, 47.8)	63

Female Sprague–Dawley rats were fed AIN-76A diet or 0.5% clofibrate in AIN-76A diet for 2 weeks. Esterase activity was measured by quantifying the metabolism of estradiol-oleoyl ester to estradiol as described in Section 2. The data for fat were presented earlier [5] and are included here for comparative purposes. Each value is the mean ± SEM obtained from five rats or the mean from two pooled samples (each obtained from five rats).

* $P = 0.05$, in comparison with control diet fed group.

** $P < 0.001$, in comparison with the control diet fed group.

esterase activity. Fat tissue had higher esterase activity (per mg protein) for the hydrolysis of the oleoyl ester of estradiol than liver, and the esterase activity (mean of microsomal and cytosolic activity) in both tissues was increased 70–107% by clofibrate administration (Table 3). A smaller clofibrate-induced increase in esterase activity (~40%) was observed in the mammary glands (Table 3).

4. Discussion

4.1. Stimulatory effect of clofibrate administration on the liver microsomal esterification of steroid hormones

Treatment of rats with clofibrate causes a many-fold increase in the liver microsomal esterification of estradiol with several fatty acids [1]. The fatty acid esters of estradiol are very lipophilic, have long half-lives, and would be expected to concentrate in fatty tissues such as the mammary glands and brain where they may undergo hydrolysis to the active hormone. In accord with this concept, treatment of rats with clofibrate had a selective stimulatory effect on the action of estradiol in the mammary glands but not in the uterus [5]. In the present study, we found that treatment of rats with clofibrate also stimulated the liver microsomal esterification of testosterone, dehydroepiandrosterone, pregnenolone, and corticosterone to their respective fatty acid esters. The physiological significance of enhanced esterification of these steroids is not known.

Estradiol has hydroxyl groups at both the 3- and 17-positions, but esterification occurs only at the nonphenolic 17-position [7]. When estrone (having a phenolic hydroxyl group at the 3-position) was incubated with liver microsomes and fatty acyl-CoA, no esterified metabolite was formed (Fig. 2, Table 1). This is in agreement with earlier studies indicating that the acyltransferase esterifies estradiol specifically at the nonphenolic 17β-hydroxyl group and that estrone is not esterified. Testosterone is also esterified at the 17-hydroxyl group. Considering the structural similarities between estradiol and testosterone, their similar microsomal esterification activities *in vitro*, their similar degrees of induction of the microsomal esterification activities, and the fact that testosterone is a competitive inhibitor of estradiol esterification [8], we suggest that the acyl-CoA:steroid acyltransferase that esterifies estradiol is probably the same enzyme that also esterifies testosterone.

Relatively high concentrations of fatty acid esters of testosterone have been found in fatty tissues of male rats [9], which is in accord with the high lipophilicity of these esters. Upon castration, testosterone levels in the fat disappeared completely within 6 hr, but a measurable fall in testosterone esters was not observed until 48 hr after castration [9]. Similar to what was observed for the estradiol fatty acid esters, it was suggested that testosterone fatty acid esters are extremely potent and long-lived androgens when compared with the parent steroid [9]. The results of our studies indicated a stimulatory effect of clofibrate administration on the esterification of testosterone by liver microsomes, and additional studies are needed to explore the biological importance of increased formation of testosterone fatty acid esters by clofibrate treatment.

Pregnenolone and dehydroepiandrosterone are Δ^5-3β-hydroxysteroids, having a nonphenolic hydroxyl group at the 3β-position. Acyl-CoA:steroid acyltransferase that catalyzes the esterification of these steroids at the 3-position has been found in several rat tissues, including brain, liver, kidney, and adrenal gland [10]. In the present study, we found that Δ^5-3β-hydroxysteroid acyltransferase activity (towards pregnenolone and dehydroepiandrosterone) in rat liver microsomes was comparable to the acyl-CoA:estradiol acyltransferase activity, and both enzyme activities were induced markedly by clofibrate administration (Table 1). An earlier study showed that pregnenolone and dehydroepiandrosterone competitively inhibit the esterification of estradiol [8]. These results suggest that the liver microsomal acyl-CoA acyltransferase that catalyzes the esterification of estradiol at the nonphenolic 17-hydroxyl group may be the same enzyme that catalyzes the esterification of pregnenolone and dehydroepiandrosterone at the nonphenolic 3-hydroxyl group.

Pregnenolone and dehydroepiandrosterone, as well as their sulfate and fatty acid ester derivatives, are more abundant in the brain than in other tissues [11], and these compounds have been called ''neurosteroids'' [12]. Although, relatively high levels of esterified pregnenolone and dehydroepiandrosterone have been extracted from rat brain [13], the role of these esterified steroids in the brain is still not clear but suggests that they may be important in modulating neuronal and behavioral function. In the present study, we found that clofibrate administration stimulated the esterification of pregnenolone and dehydroepiandrosterone by liver microsomes. It will be of interest to explore the biological consequences of an altered esterification of these neurosteroids *in vivo*.

Corticosterone is esterified at the 21-hydroxyl group [14]. The rate of *in vitro* esterification of corticosterone was only about 1/20th of that for the esterification of estradiol (Table 1). An earlier study showed that with an *in vitro* tissue culture system using small pieces of tissue, the fatty acid composition of esterified corticosterone metabolites in several tissues was very different from the fatty acid composition of esterified estradiol in the same tissues, and the relative formation ratio of corticosterone esters to estradiol esters differed more than 100-fold between tissues [15]. These observations suggest that the acyltransferase that synthesizes C-17 esters of estradiol is different from the one that synthesizes C-21 esters of corticosterone. Definitive evidence for differences in acyltransferases requires their isolation and characterization. Differences

in the ratios of estradiol esters and corticosterone esters between various tissues could also be explained by differences in esterase activities for the hydrolysis of the different steroid fatty acid esters.

In contrast to the prolonged estrogenic activity of estradiol fatty acid esters, corticosterone-21-stearoyl ester, which was studied as a representative glucocorticoid ester, did not have enhanced or prolonged activity when compared with corticosterone [16]. This difference between estradiol and corticosterone fatty acid esters can be explained by the fact that corticosterone-21-stearoyl ester is hydrolyzed at a much faster rate than estradiol fatty acid esters [16], and these observations may also explain why corticosterone is apparently esterified at a much lower rate than estradiol, testosterone, pregnenolone, and dehydroepiandrosterone (Table 1). The rapid desterification of corticosterone fatty acid esters may explain why the esterification of corticosterone appears not to result in an increased biological half-life, as occurs for estradiol and testosterone. The physiological role of corticosterone fatty acid esters is not known.

4.2. Esterification and deesterification of steroid hormones by liver and extrahepatic tissues

The acyl-CoA:estradiol acyltransferase that catalyzes the esterification of estradiol with fatty acids is present in liver and extrahepatic tissues of rats. Among the tissues examined, brain had the highest acyltransferase activity for estradiol esterification, and no significant differences were observed between the cortex, cerebellum, and brain stem (Table 2). Similar results were observed in a study on Δ^5-3β-hydroxysteroid acyltransferase, in which the acyltransferase for the esterification of Δ^5-3β-hydroxysteroids such as pregnenolone and dehydroepiandrosterone was significantly higher in the brain than in other tissues [10]. The high acyltransferase activity for estradiol esterification in the brain is of considerable interest, and suggests the presence of an elevated level of estradiol fatty acid esters in the brain. The biological role of these estradiol esters in the brain is not known.

An earlier study determined the *in vitro* esterification of estradiol by various tissues from rats [15]. Each tissue (~500 mg) was cut into 2–4 mm^3 pieces, and placed in Hanks' medium. [^{3}H]Estradiol (0.1 μM) was added to the tissue culture medium and incubated at 37° in an atmosphere of 95% O_2 and 5% CO_2. Tissues were then extracted for quantification of estradiol fatty acid esters. Of the tissues examined, spleen synthesized the most estradiol fatty acid esters, followed by lung, uterus, mammary glands, kidney, brain, and liver. Fat was the least active of the several tissues examined for estradiol esterification [15]. Differences between our study and the earlier study may have resulted from the two different incubation systems used to measure the *in vitro* esterification of estradiol or variations in esterase activity among the different tissues. The tissue culture study by Pahuja and Hochberg [15] used small pieces of tissue, and the formation of estradiol fatty acid esters depended upon the tissue acyltransferase level, cofactor concentration, and esterase activity. For the *in vitro* microsomal assays in our study, acyltransferase activity was evaluated in the presence of optimal cofactor levels.

Administration of clofibrate to rats had a strong stimulatory effect on the formation of estradiol fatty acid esters in liver but had little or no effect on estradiol esterification in extrahepatic tissues (Table 1). As shown in other studies, extrahepatic tissues were more refractory to induction of microsomal and peroxisomal fatty acid oxidizing enzymes by peroxisome proliferators than the liver [17,18]. Since the availability of peroxisome proliferators in these tissues may not be a critical factor [17], differences in the magnitude of induction in different tissues implies tissue-specific regulatory controls.

In a recent study, we found that the induction of liver microsomal acyl-CoA:estradiol acyltransferase by a prototypical peroxisome proliferator is dependent upon peroxisome proliferator-activated receptor alpha (PPARα) [19]. It is known that PPARα is expressed at different levels in different rat tissues [20]. Liver has the highest level of PPARα mRNA expression, which may account for the high sensitivity of liver to the induction of acyltransferase for estradiol esterification. In kidney, PPARα is expressed at a very high level in proximal tubules, but other parts of the kidney (glomeruli, Henle's loops, distal lobules, collecting tubes) have little or no PPARα expression. Thus, it is possible that certain cell types in the kidney are very sensitive to peroxisome proliferators but are masked during whole-tissue enzyme activity analysis. Brain cortex, cerebellum, and brain stem have little or no expression of PPARα, and we did not observe enzyme induction by clofibrate in the brain. Low levels of PPARα are expressed in the uterus and in fat, and clofibrate administration did not increase acyl-CoA:estradiol acyltransferase activity significantly in these tissues. Our results suggest a correlation between PPARα tissue levels and sensitivity to induction of acyl-CoA:estradiol acyltransferase among different tissues. In a recent study, we found that treatment of wild-type mice with the potent peroxisome proliferator Wy-14,643 stimulated the liver microsomal esterification of estradiol and testosterone, but this did not occur in PPARα knockout mice [19]. Our results suggest that the induction of acyl-CoA:steroid acyltransferase by clofibrate and Wy-14,643 is dependent upon PPARα.

In an additional study, we determined the effect of clofibrate administration on esterase activity in liver, fat, and mammary gland. Carboxylesterase catalyzes the hydrolysis of a large number of xenobiotics, and many isozymes have been isolated and characterized. The induction of carboxylesterase by peroxisome proliferators is substrate dependent [21,22]. In this study, we determined

esterase activity for the hydrolysis of an estradiol fatty acid ester by using estradiol-oleoyl ester as the substrate. The results of our study showed that treatment of rats with clofibrate increased esterase activity by about 40% in the mammary glands and by 70–107% in fat and liver (Table 3), suggesting that clofibrate administration may stimulate the release of hormonally active estradiol from their fatty acid esters that reach lipophilic target tissues.

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